

Resistance of *Pseudomonas pseudomallei* Growing as a Biofilm on Silastic Discs to Ceftazidime and Co-Trimoxazole

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We have examined the resistance of *Pseudomonas pseudomallei* biofilm cells to ceftazidime and co-trimoxazole. A large number of these biofilm cells remained viable at 12 and at 24 h, except in the biofilm treated with 200 times the MIC of ceftazidime. The inherent resistance of *P. pseudomallei* biofilms to conventional antibiotics may explain the lack of success in the treatment of the chronic manifestations of this bacterial infection.

Since the promulgation of the general biofilm theory in 1978 (3), it has become obvious that growth in biofilms allows bacteria a large measure of protection from antibacterial agents (2). *Pseudomonas pseudomallei* is of special interest in this respect because it grows preferentially in microcolonies and biofilms, both in vitro and in vivo in an animal model (12a), and because the disease that it causes (melioidosis) manifests a chronic phase (9, 12) that is especially refractory to antibiotic chemotherapy (1, 13). For these reasons, we obtained cultures of this pathogen from Southeast Asia, where melioidosis is endemic, and we have undertaken to determine the degree to which its biofilm cells are resistant to the antibiotics (ceftazidime [CTZ] and co-trimoxazole [SXT]) most commonly used in its treatment.

Biofilms were formed on the silastic surface of the sampling plugs of a modified Robbins device (11) for 16 h and treated with 0, 25, 50, 100, and 200 times the MICs of CTZ and SXT. Samples for colony counting and scanning electron microscopy were taken at 0, 12, and 24 h.

The silastic discs of the modified Robbins device were colonized very rapidly by cells of *P. pseudomallei* to produce sessile populations of 8.8×10^4 cells per cm^2 at 1 h and a stable biofilm population of 1.9×10^6 to 6.5×10^6 cells per cm^2 after 16 h, by which time the silastic surface was occluded by a confluent biofilm containing the bacterial cells partially buried in the dehydrating condensed residue of their glycocalyx (Fig. 1A).

The MICs of CTZ and SXT for the planktonic cells of *P. pseudomallei* taken from the batch cultures and used to inoculate the experimental system were 4 and 40 $\mu\text{g/ml}$, respectively, while the MBCs were 8 and 3,000 $\mu\text{g/ml}$, respectively. When *P. pseudomallei* biofilms were treated with 0, 25, 50, 100, and 200 times the MICs of CTZ and SXT in Mueller-Hinton broth in the sessile minimum biofilm eliminating concentration testing device, a large number of cells remained viable at 12 h and an almost equally large number were still active at 24 h, except in the biofilm treated with 200 times the MIC of CTZ, in which only 8.4×10^3 cells per cm^2 survived at 24 h (Table 1). When these CTZ- and SXT-treated biofilms were examined by scanning electron microscopy, the CTZ-treated biofilm showed marked elongation of *P. pseudomallei* cells (Fig. 1B), while the SXT-treated biofilm did not show this elongation and even showed

cell division (Fig. 1C). When the MICs of CZT and SXT were determined for dispersed cells recovered from the biofilms on the silastic discs, the MICs for these two antimicrobial agents were found to be 4 and 40 $\mu\text{g/ml}$, respectively, the same values as for planktonic cells.

The inherent resistance of biofilm bacteria to antibiotics is currently believed to be caused by a combination of the diffusional resistance posed by the biofilm matrix and by the profound physiological differences between planktonic and sessile cells of the same bacterial species (4).

Biofilms and microcolonies essentially identical to those seen on the colonized surfaces of medical devices have now been described for non-device-related human bacterial infections such as osteomyelitis (8), endocarditis (7), prostatitis (10), cystic fibrosis pneumonia (5), and melioidosis (12a). These chronic bacterial infections are also associated with frequent recurrence following antibiotic therapy, and with the exception of native valve endocarditis (6), all are considered to be highly refractory to antimicrobial chemotherapy.

The chronic manifestation of the *P. pseudomallei* infections that cause significant morbidity and mortality in Southeast Asia is only very rarely resolved by antibiotic therapy (12), and despite apparent bacterial susceptibility and continuous antimicrobial chemotherapy, many chronic cases have been documented over the course of one or more decades (9). CTZ and SXT are the agents commonly used to treat melioidosis (14), and the present study has shown that their very encouraging MICs against planktonic cells of *P. pseudomallei* explain their combined clinical efficacy against the acute phase of this disease, in which planktonic bacteria overwhelm the lung and the circulation (12a). However, the very high minimum biofilm eliminating concentration found when we tested these agents against biofilm cells of *P. pseudomallei* also explains their lack of success in the treatment of the chronic manifestations of this notably protean bacterial infection (12a).

A wide variety of antibiotics could now be tested against *P. pseudomallei* biofilms to determine which agents are naturally effective against these biofilms so that the efficacy of antibiotic treatment of the chronic phase of this disease could be improved; however, the general efficacy of antibiotic treatment of biofilm-related chronic infections will be best served by the development of new classes of antibiotics that penetrate biofilms and kill sessile bacteria.

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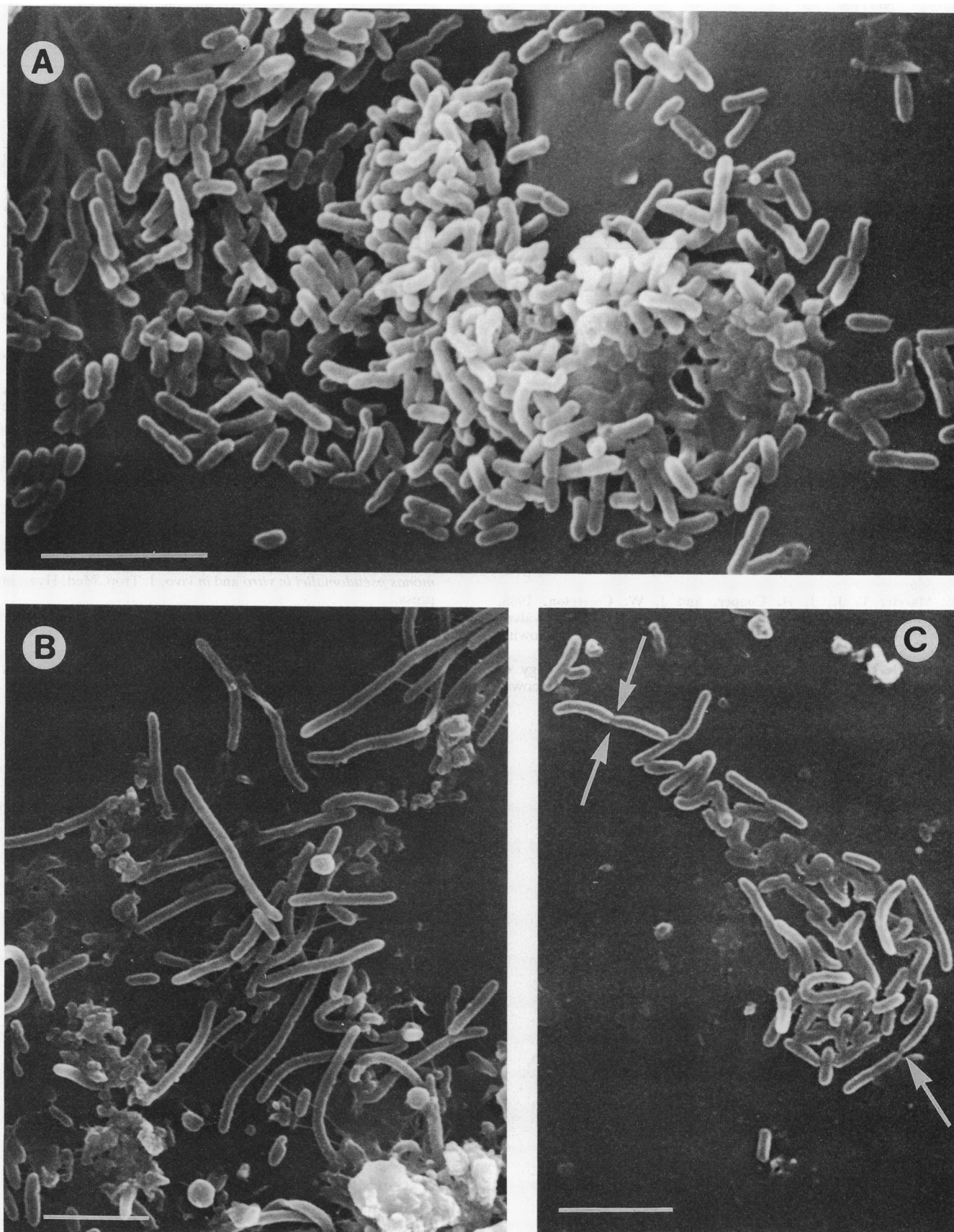


FIG. 1. Scanning electron micrographs of the silastic disc after contact with *P. pseudomallei* in liquid modified Vogel and Bonner media for 16 h (A), biofilm treated with 100 times the MIC for CTZ for 12 h (B), and biofilm treated with 200 times the MIC of SXT for 48 h (C). Note the elongated bacterial cells (B) and the persistence of cell division (arrows) (C). Bars, 5 μ m.

TABLE 1. Effects of high concentrations of CTZ and SXT on *P. pseudomallei* cells growing within biofilms on silastic discs

CTZ concn (μg/ml)	SXT concn (μg/ml)	Multiple of MIC	No. of viable cells/cm ²				
			0 h	CTZ		SXT	
				12 h	24 h	12 h	24 h
0	0	0	1.9×10^6	7.0×10^8	1.1×10^7	2.5×10^6	1.1×10^7
100	1,000	25	1.9×10^6	1.4×10^6	1.3×10^5	1.5×10^6	3.8×10^5
200	2,000	50	1.9×10^6	3.5×10^5	1.4×10^6	1.3×10^6	6.5×10^5
400	4,000	100	6.5×10^5	2.3×10^6	3.3×10^4	2.0×10^5	7.3×10^4
800	8,000	200	6.5×10^5	1.0×10^5	8.4×10^3	1.7×10^5	6.1×10^4

REFERENCES

1. Aswapokee, N. 1989. Antimicrobial treatment of melioidosis perspective, p. 230–232. In S. Punyagupta, T. Sirirsathana, and B. Stapatayavong (ed.), *Melioidosis*. Bangkok Medical Publisher, Bangkok, Thailand.
2. Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie. 1987. Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol.* **41**:435–464.
3. Costerton, J. W., G. G. Geesey, and K. J. Cheng. 1978. How bacteria stick. *Sci. Am.* **238**:86–95.
4. Hoyle, B. D., J. Jass, and J. W. Costerton. 1990. The biofilm glycocalyx as a resistance factor. *J. Antimicrob. Chemother.* **26**:1–6.
5. Lam, J., R. Chan, K. Lam, and J. W. Costerton. 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect. Immun.* **28**:546–556.
6. Marrie, T. J., J. H. Cooper, and J. W. Costerton. 1988. Ultrastructure of cardiac bacterial vegetations on native valves with emphasis on alterations in bacterial morphology following antibiotic treatment. *Can. J. Cardiol.* **3**:275–280.
7. Marrie, T. J., and J. W. Costerton. 1984. Morphology of bacterial attachment to cardiac pacemaker leads and power packs. *J. Clin. Microbiol.* **19**:911–914.
8. Marrie, T. J., and J. W. Costerton. 1985. Mode of growth of bacterial pathogens in chronic polymicrobial human osteomyelitis. *J. Clin. Microbiol.* **22**:924–933.
9. Morrison, R. E., A. S. Lamb, D. B. Craig, and W. M. Johnson. 1988. Melioidosis: a reminder. *Am. J. Med.* **84**:965–967.
10. Nickel, J. C., M. E. Olson, A. Barabas, H. Benediktsson, M. K. Dasgupta, and J. W. Costerton. 1990. Pathogenesis of chronic bacterial prostatitis in an animal model. *Br. J. Urol.* **65**:47–54.
11. Nickel, J. C., I. Ruseska, J. B. Wright, and J. W. Costerton. 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob. Agents Chemother.* **27**:619–624.
12. Punyagupta, S. 1983. Melioidosis: the great imitator. *Ramathibodi Med. J.* **6**:147–153.
- 12a. Vorachit, M., K. Lam, P. Jayanetra, and J. W. Costerton. Electron microscopy study of the mode of growth of *Pseudomonas pseudomallei* *in vitro* and *in vivo*. *J. Trop. Med. Hyg.*, in press.
13. White, N. J., and D. A. B. Dance. 1988. Clinical and laboratory studies of malaria and melioidosis. *Trans. R. Soc. Trop. Med. Hyg.* **82**:15–20.
14. White, N. J., D. A. B. Dance, W. Chaowaagul, Y. Wattanagoon, V. Wuthiekanun, and N. Pitakwatchara. 1989. Halving of mortality of severe melioidosis by ceftazidime. *Lancet* **ii**:697–701.